Oxidative Stress, Nitric Oxide, and Vascular Disease

Jamie Y. Jeremy, M.Sc., Ph.D., F.R.S.H.,* Anthony P. Yim, F.R.C.S.,† Song Wan, F.R.C.S.,† and Gianni D. Angelini, F.R.C.S.*

**Bristol Heart Institute, The University of Bristol, United Kingdom, and †Department of Surgery, Prince of Wales Hospital, Chinese University of Hong Kong, China*

ABSTRACT **Superoxide (O_2^-) is a key risk factor for cardiovascular disease (CVD), including atherogenesis, reperfusion injury, angina, restenosis following balloon angioplasty, and vein graft failure. Axiomatically, O_2^- reacts with nitric oxide (NO) to form peroxynitrite (ONOO) resulting in a depletion of endogenous vascular NO, which is now firmly associated with CVD. Furthermore, risk factors for CVD, in particular diabetes mellitus (DM), dyslipidemia, and hyperhomocysteinemia are all associated with oxidative stress OS. Antioxidant therapies, including the gene transfer of antioxidant enzymes, are potentially valuable in the treatment of CVD.** *(J Card Surg 2002;17:324-327)*

Oxidative stress (OS) is a condition in which cells are exposed to excessive levels of O_2 or derivatives of O_2 (reactive oxygen species [ROS]), principal among which is superoxide (O_2^-).[1-3] Protective systems that remove excess O_2^- include superoxide dismutase (SOD; breaks down O_2^- to H_2O_2) and catalase (breaks down H_2O_2 to H_2O).[1-3] Thus, OS can be considered to be the upregulation of O_2^- generating systems coupled with a down regulation of protective systems.

O_2^- elicits a number of pathogenic effects including: the promotion of lipid peroxidation, the proliferation of VSMCs, apoptosis of endothelial cells and vasoconstriction.[1-3] However, the reaction $O_2^- + NO \rightarrow$ peroxynitrite (ONOO) is axiomatic in CVD since this effectively diminishes NO levels which in turn is associated with angina, atherogenesis, hypertension, ARDS, diabetic angiopathy, vein graft disease, reperfusion injury, and restensosis following balloon injury.[2,4-6]

BLOOD CELLS AND SUPEROXIDE

O_2^- acts at both the blood cell and vascular tissue level, both of which interact to produce pathology. First, platelets, neutrophils, monocytes, lymphocytes, and erythrocytes all possess the capacity to generate O_2^-.[7-9] Neutrophils release large amounts of O_2^-[7] and co-adhere with platelets and monocytes.[10-12] Because O_2^- promotes adhesion molecule expression in blood and vascular cells,[13,14] this is the primary event in OS mediated vasculopathy. Neutrophils release substances that also promote the demise of endothelial cells.[15] The resultant denudation results in further aggressive adhesion of platelets, neutrophils, and monocytes, creating a self-perpetuating cascade. The release of O_2^- from these blood cell complexes, immediately negates endothelial NO availability.[4-6] Since diminished NO formation promotes adhesion molecule expression[4,6] this would further exacerbate this ongoing pathogenic cascade.

Platelets, neutrophils and monocytes generate vasoconstrictors, cytokines, peptide growth

This study was supported by Research Grant Council Earmarked grants (CUHK 4255/97M, CUHK 4310/99M and CUHK 4091/00M), Hong Kong.

Presented at the First International Symposium of Cardiovascular Science: From Bench to Bedside. November 23-24, 2001, Hong Kong.

Address for correspondence: Jamie Y. Jeremy, Ph.D., Bristol Heart Institute, Bristol Royal Infirmary, Bristol, BS2 8HW. Fax: 0117-929-9737; e-mail: j.y.jeremy@bristol.ac.uk

factors, and thrombogens.[8] Thus, apart from promoting acute vasoconstriction, thrombogenesis, and inflammation, these events may also trigger longer term events, including the invasion of monocytes (progenitors of the macrophage and foam cell) and the proliferation of VSMCs.[8]

CVD, RISK FACTORS, AND SUPEROXIDE

Risk factors for CVD (e.g., diabetes mellitus, dyslipidemia, copper, smoking, and homocysteinemia) are all associated with hyperactive neutrophils, platelets, and monocytes, increased adhesion molecule expression, diminished NO formation, and of course, OS.[4-6,8,16-18] Homocysteine auto-oxidizes to generate O_2^- to negate NO bioactivity,[18] an effect augmented by copper.[19-21] Physiological concentrations of homocysteine and copper markedly inhibit NO formation in aortas of diabetic animals,[22,23] indicating a susceptibility to OS-promoting factors in diabetes. Atherosclerosis and the above risk factors are also associated with increased vascular O_2^- formation and decreased NO bioavailability.[3-6] The major enzymatic source of O_2^- in blood vessels is NADPH oxidase, which is overexpressed in the vasculature atherosclerotic and diabetic animals.[24]

OXIDANT STRESS AND CARDIAC SURGERY

OS is associated with thromboembolic and inflammatory complications immediately after surgery.[25] Coronary artery bypass graft surgery (CABG) using cardiopulmonary bypass (CPB) are associated with an enhanced acute phase response (i.e an acute inflammatory-immune response) which is characterized by a marked increase in blood levels of cytokines.[26] Neutrophils and platelets also adhere in large numbers to recently implanted vein grafts.[8] It was suggested that this early adhesion on neutrophils may elicit the release of growth factors and cytokines that may trigger/enhance the early proliferation of VSMCs in vein grafts, hence promulgating the formation of a neointimal, the central lesion for vein graft failure.[8] It is tempting to speculate, therefore, that adherent blood cells through their local release of cytokines may enhance endogenous oxidant stress in saphenous vein grafts. As was discussed, homocysteine and copper interact to generate O_2^-, an effect is markedly augmented in DM. We found a striking increase in plasma levels of HCy and copper after CABG that persisted for up to 6 weeks after surgery.[27] It follows, therefore, that these changes could contribute to vein graft disease through an augmentation of OS.

ANTIOXIDANT THERAPY

The number and range of orally active antioxidants is enormous and any studies in animal models of vascular disease (and the risk factors thereof) have demonstrated that antioxidants reduce OS and associated vasculopathy.[3] These antioxidants include vitamins C and E, probucol allopurinol, SOD mimetics, NADPH oxidase inhibitors, desferrioxamine, and penicillamine. Interestingly, the gene transfer of SOD reversed endothelial dysfunction in diabetic rabbits.[28]

In man, fewer cardiac events in patients taking vitamin C have been reported[29] as well as improving endothelium-dependent vasodilation in diabetic and homocysteinemic patients, indicating an inhibitory effect on the O_2^--NO axis.[30,31] Vitamin C administration also reduces cytokine levels, indicating an impact on leukocyte function.[32] In large population studies significant inverse associations between cardiac events and vitamin E intake have been reported.[33-35] In turn, antioxidants have shown promise in reducing OS in patients undergoing cardiac surgery.[36]

CONCLUSIONS

Although it is clear that OS plays a pivotal role in both acute and chronic CVD, the benefits of antioxidant therapies is ambivalent and certainly more clinical trials are required to clarify the area. Acute inflammatory OS (associated with cardiac surgery) constitutes an aggressive and local release of O_2^- and other ROS which may simply overwhelm acute antioxidant therapy. However, in surgery especially, the administration of antioxidants prior to procedures may combat OS precipitated by the procedure. Other antioxidants may be more useful in preventing more long term pathologies (e.g. late vein graft failure). Secondly, the distribution of OS between the blood and tissue compartments may be crucial to the efficacy of antioxidant therapy. Any given antioxidant may be effective in preventing O_2^- damage by leukocyte-platelet complexes but be ineffective in prevent-

ing OS in endothelial or VSMCs, and vice versa. Thus, the use of adjuvant preparations could be deployed to create broad spectrum antioxidant regimes. Notwithstanding these considerations, more studies on OS and antioxidant effects on the pathophysiology of CVD using different animal models is also mandatory, since the mechanisms underlying this complex area are still far from being fully understood.

REFERENCES

1. Betteridge DJ: What is oxidative stress? Metabolism 2000;49:3-8.
2. Kojda G, Harrison D: Interaction between NO and reactive oxygen species: Pathological importance in atherosclerosis, hypertension, diabetes, and heart failure. Cardiovasc Res 1999;43:562-571.
3. Young IS, Woodside JV: Antioxidants in health and disease. J Clin Pathol 2001;54:176-186.
4. Napoli C, de Nigris F, Palinski W: Multiple role of reactive oxygen species in the arterial wall. J Cell Biochem 2001;82:674-682.
5. Napoli C, Ignarro LJ: Nitric oxide and atherosclerosis. Nitric Oxide 2001;5:88-97.
6. Jeremy JY, Rowe D, Emsley AM, et al: Nitric oxide and vascular smooth muscle cell proliferation. Cardiovasc Res 1999;43:658-665.
7. Babior B: Phagocytes and oxidative stress. Am J Med 2000;109:33-44.
8. Jeremy JY, Mehta D, Bryan AJ, et al: Platelets and saphenous vein graft failure following coronary artery bypass graft surgery. Platelets 1997;8:295-309.
9. Stuart-Smith K, Jeremy JY: Microvessel damage in acute respiratory distress syndrome: The answer may not be NO. Br J Anaesth 2001;87:272-279.
10. Ott I, Neumann F-J, Gawaz M, et al: Increased neutrophil—platelet adhesion in patients with unstable angina. Circulation 1996;94:1239-1246.
11. de Gaetano G, Cerletti C, Evangelista V: Recent advances in platelet-polymorphonuclear leukocyte interaction. Haemostasis 1999;29:41-49.
12. Li N, Hu H, Lindqvist M, et al: Platelet-leukocyte cross talk in whole blood. Arterioscler Thromb Vasc Biol 2000;20:2702-2708.
13. Nagata K, Tsuji T, Todoroki K, et al: Activated platelets induce superoxide anion release by monocytes and neutrophils through P-selectin (CD62). J Immunol 1993;151:3267-3273.
14. Practico D, Juliano L, Alessandri C, et al: Polymorphonuclear leukocyte derived O_2^- reactive species activate primed platelet in human whole blood. Am J Physiol 1993;264:H1582-1587.
15. Abdu TA, Elhadd T, Pfeifer M, et al: Endothelial dysfunction in endocrine disease. Trends Endocrinol Metab 2001;12:257-265.
16. Blann AD, McCollum CN: Circulating endothelial cell/leukocyte adhesion molecules in atherosclerosis. Thrombos Haemostas 1995;72:151-154.
17. Gearing AJH, Newman W: Circulating adhesion molecules in disease. Immunol Today 1993;14:506-512.
18. Emsley AM, Plane F, Jackson CL, et al: Oxidant stress, nitric oxide and transition metals in homocysteinaemic angiopathy: novel mechanisms. Vasc Dis 1998;1:66-72.
19. Khan M, Thompson CS, Emsley A, et al: Homocysteine and copper interact to markedly inhibit the relaxation of the rabbit corpus cavernosum. New risk factors for angiopathic erectile dysfunction? Br J Urol 1999;84:720-724.
20. Emsley AM, Jeremy JY, Gomes GN, et al: Investigation of the inhibitory effects of homocysteine and copper on nitric oxide-mediated relaxation of rat isolated aorta. Br J Pharmacol 1999;126:1034-1040.
21. Ford ES: Serum copper concentrations and coronary heart disease among US adults. Am J Epidemiol 2000;151:1182-1188.
22. Jeremy JY, Shukla N, Marshman N, et al: Homocysteine augments impaired endothelium dependent relaxation and cGMP formation in aortae of diabetic rats. Atherosclerosis 2000;151: 15.
23. Shukla N, Taberner P, Thompson CS, et al: Homocysteine further augments impaired acetylcholine-stimulated relaxation and cyclic GMP formation in aortae from diabetic rabbits. Br J Pharmacol 2000;131:SP.
24. Griendling KK, Sorescu D, Ushio-Fukai M: NAP(P)H oxidase. Role in cardiovascular biology and disease. Circ Res 2000;86:494-501.
25. Azevedo LC, Pedro MA, Souza LC, et al: Oxidative stress as a signalling mechanism of the vascular response to injury: The redox hypothesis of restenosis. Cardiovasc Res 2000;47:436-445.
26. Tonnesen E, Christensen VB, Toft P: The role of cytokines in cardiac surgery. Int J Cardiol 1996;26:S1-S10.
27. Jeremy JY, Lotto A, Day A, et al: Homocysteine, copper and caeruloplasmin in patients undergoing coronary artery bypass graft surgery. Atherosclerosis 2000;151:108.
28. Zanetti M, Sato J, Katusic ZS, et al: Gene transfer of superoxide dismutase isoforms reverses endothelial dysfunction in diabetic rabbit aorta. Am J Physiol 2001;280:H2523-H2523.
29. Losonczy KG, Harris TB, Havlik RJ: Vitamin E and vitamin C supplement use and risk of all-cause and coronary heart disease mortality in older persons: The Established Populations for Epidemiologic Studies of the Elderly. Am J Clin Nutr 1996;64:190-196.

30. Ting HH, Timimi FK, Boles KS, et al: Vitamin C improves endothelium-dependent vasodilation in patients with non-insulin dependent diabetes mellitus J Clin Invest 1996;97:22-28.
31. Chambers JC, McGregor A, Jean-Marie J, et al: Demonstration of rapid onset vascular endothelial dysfunction after hyperhomocystainaemia: An effect reversible with vitamin C therapy. Circulation 1999;99:1156-1160.
32. Petersen EW, Ostrowski K, Ibfelt T, et al: Effect of vitamin supplementation on cytokine response and on muscle damage after strenuous exercise. Am J Physiol Cell Physiol 2001;280: C1570-C1576.
33. Rimm EB, Stampfer MJ, Ascherio A, et al: Vitamin E consumption and the risk of coronary heart disease in men. N Encl. J Med 1993;328:1450-1456.
34. Sampler AJ, Heineken CH, Manson JE, et al: Vitamin E consumption and the risk of coronary disease in women. N Engl J Med 1993;28:1444-1449.
35. Stephens NG, Parsons A, Schofields PM, et al: Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study (CHAOS). Lancet 1996;347:781-786.
36. McColl AJ, Feeble T, Hadjinikolaou L, et al: Plasma antioxidants: Evidence for a protective role against reactive oxygen species following cardiac surgery. Ann Clin Biochem 1999;36:683-684.

0022-5347/01/1661-0341/0
THE JOURNAL OF UROLOGY®
Copyright © 2001 by AMERICAN UROLOGICAL ASSOCIATION, INC.®

Vol. 166, 341–346, July 2001
Printed in U.S.A.

PROTECTIVE EFFECT OF VITAMIN E ON THE RESPONSE OF THE RABBIT BLADDER TO PARTIAL OUTLET OBSTRUCTION

MITESH H. PAREKH, ROBERT LOBEL, LAURA J. O'CONNOR, ROBERT E. LEGGETT AND ROBERT M. LEVIN

From the Department of Uro-Gynecology, Albany Medical College, Department of Basic and Pharmaceutical Sciences, Albany College of Pharmacy and Stratton Veterans Affairs Medical Center, Albany, New York

ABSTRACT

Purpose: There is increasing evidence that ischemia/reperfusion is a major etiological factor in the progression of bladder dysfunction after partial outlet obstruction. If this evidence is correct, treatment with an antioxidant should be beneficial in rabbits subjected to partial outlet obstruction. We designed the current study to determine if diets high in α-tocopherol protected the rabbit bladder against dysfunction induced by partial outlet obstruction.

Materials and Methods: A total of 32 rabbits were separated into 4 groups of 8. Groups 1 and 2 were placed on a diet enriched with 1,000 IU/kg. α-tocopherol, and groups 3 and 4 were fed a regular diet containing 44 IU/kg. α-tocopherol. After 4 weeks partial outlet obstruction was created in groups 1 and 3, while groups 2 and 4 underwent sham operation. After 4 weeks of obstruction the rabbits were anesthetized and the bladders were rapidly excised. Four longitudinal strips obtained from the bladder body were used for contractility studies. The balance of the bladder body was separated between muscle and mucosa. Each section was frozen and stored at −70C for analysis of malondialdehyde as a measure of peroxidation and for α-tocopherol concentrations.

Results: Feeding rabbits a diet high in α-tocopherol resulted in significant protection against the development of contractile dysfunction after partial outlet obstruction. The protective effect of α-tocopherol was related to significantly decreased malondialdehyde and significantly increased tissue concentrations of α-tocopherol.

Conclusions: These data indicate that a major etiology of bladder dysfunction secondary to partial outlet obstruction is related to free radical generation and resultant membrane lipid peroxidation.

KEY WORDS: bladder; muscle, smooth; rabbits; ischemia; vitamin E

Benign prostatic hyperplasia (BPH) is a common medical problem. More than 80% of males 50 years old or older have some degree of bladder outlet obstruction secondary to BPH.[1–3] To understand the effects of outlet obstruction on bladder morphology, physiology and pharmacology animal models of obstruction have been developed using several species, including the rat, rabbit, guinea pig and pig.[4,5] Although there are marked differences in bladder size, capacity, compliance, physiology and pharmacology among these species, responses to outlet obstruction have many common characteristics. Common to virtually all models of partial outlet obstruction are an increase in bladder mass, and progressive decreases in bladder contractile function and ability to empty.

There is increasing evidence that ischemia/reperfusion is a major etiological factor in the progression of bladder dysfunction associated with experimental partial outlet obstruction.[6–9] Our working hypothesis was that partial outlet obstruction induces an increase in bladder mass and wall thickness.[10,11] Increased bladder wall thickness results in cyclical ischemia/reperfusion during every micturition and uninhibited contraction.[9,12] Blood flow and tissue oxygen tension increase after micturition or uninhibited contraction. Reperfusion and re-oxygenation generate reactive oxygen species that cause the lipid peroxidation of cellular membranes. The result is membrane damage induced by ischemia directly and indirectly via lipid peroxidation.[13,14] This continuing membrane damage underlies the progressive bladder dysfunction associated with partial outlet obstruction.

In addition to contractile dysfunction induced by partial outlet obstruction that results in decreased voiding pressure, decreased flow and increased post-void residual urine,[4,5,10] ischemic injury to the mucosa causes increased mucosal permeability[15] and activation of the sensory nerves, which may be related to irritability symptoms such as urgency, frequency and urge incontinence. Thus, ischemic injury to muscle and mucosal elements are related to bladder dysfunction secondary to partial outlet obstruction. If this hypothesis is correct, treatment with an antioxidant should be beneficial in rabbits subjected to partial outlet obstruction. Our study was designed to determine whether diets high in α-tocopherol protected the rabbit bladder against dysfunction induced by partial outlet obstruction.

α-Tocopherol is lipid soluble potent antioxidant. Of the of 4 tocopherols (α, β, γ and δ) in the family α-tocopherol is the most abundant in nature and the most potent based on various tests.[16] In addition to its antioxidant actions, studies have indicated a number of further biological activities, including the inhibition of protein kinase C and activation of phosphoprotein phosphatase 2A.[16]

MATERIALS AND METHODS

Preliminary experiment. A total of 12 rabbits were separated into 2 groups of 6. The rabbits in group 1 were fed a normal control diet containing 44 IU α-tocopherol per kg. and

Accepted for publication January 5, 2001.
Supported in part by grants from the Veterans Administration Medical Center and National Institutes of Health Grants RO-1-DK 26508, RO-1-DK 47949 and RO-1-DK.

those in group 2 were fed a normal diet supplemented with 1,000 IU/kg. α-tocopherol. Three animals per group were anesthetized with sodium pentobarbital after 4 and 6 weeks. The bladder was excised from each rabbit and homogenized. Mitochondria and microsomal preparations isolated by differential centrifugation were analyzed for α-tocopherol by fluorescent high performance liquid chromatography.

Experimental protocol. A total of 32 rabbits were separated into 4 groups of 8. Groups 1 and 2 were placed on a diet enriched with α-tocopherol, while groups 3 and 4 were fed a regular diet. After 4 weeks partial outlet obstruction was created in groups 1 and 3; while groups 2 and 4 underwent sham operation. After 4 weeks the rabbits were anesthetized and the bladders were excised. Four longitudinal strips obtained from the bladder body were used for contractility studies. The balance of the bladder body was separated between muscle and mucosa. Each section was frozen and stored at −70C for biochemical analysis.

Surgery. A standard method was used to create moderate bladder outlet obstruction in groups 1 and 3.[10] Each rabbit was sedated with 25 mg./kg. ketamine-10 mg./kg. xylazine given intramuscularly and surgical anesthesia was maintained with 25 mg./kg. sodium pentobarbital given intravenously. Under sterile conditions the bladder was catheterized and drained with an 8Fr catheter. The bladder and urethrovesical junction were exposed through a lower abdominal midline incision. After removing the perivesical fat body a 2-zero silk ligature was tied loosely around the vesical outlet approximately 1 to 2 cm. distal to the insertion of the ureters. The catheter was removed. The bladder was returned to its normal anatomical position and the wound was closed anatomically. Gentamicin (1 mg./kg.) and 0.3 mg./kg. buprenorphine hydrochloride were given intramuscularly on postoperative days 1 and 2. All obstruction procedures were performed by the same surgeon. Sham operations were performed as described but no ligature was tied.

Contractility studies. A full-thickness section of each bladder was cut into 3 longitudinal strips of approximately 1 × 1 × 12 mm. Each strip was mounted in a 15 ml. organ bath containing Tyrode's solution (124.9 mM. NaCl, 2.5 mM. KCl, 23.8 mM. $NaHCO_3$, 0.5 mM. $MgCl_2$-6 H_2O, 0.4 mM. NaH_2PO_4. H_2O, 1.8 mM. CaCl and 5.5 mM. glucose) at 37C and equilibrated with 95% O_2-5% CO_2. One end of each strip was connected by a 1-zero silk suture to an FT03 isometric force transducer (Grass Instruments, Quincy, Massachusetts), and tension changes were measured and recorded with a 7D Polygraph recorder (Grass Instruments). The analog signal was digitized using the Polyview System (Grass Instruments) and the maximal rate of tension generation was calculated for all responses. All strips were subjected to 2 gm. of initial resting tension and equilibrated for 30 minutes before initiating contractile studies.

After equilibration the bladder strips were stimulated at 3 square minute intervals with 1 msec. wave pulses applied through ring platinum electrodes at frequencies of 1, 2, 4, 8, 16 and 32 Hz. After electrical field stimulation bladder strip responses to 1 mM. adenosine triphosphate, 20 μM. carbachol (Sigma Chemical Corp., St. Louis, Missouri) and 120 mM. KCl (Fisher Scientific, Fairlawn, New Jersey) were determined. Between the applications of pharmaceutical agents the strips were washed with fresh Tyrode's solution 3 times at 15-minute intervals.

Tissue preparation. Frozen samples of bladder smooth muscle and mucosa were weighed and thawed in 1.15% KCl-0.05 M. tris buffer, pH 7.4, on ice and homogenized for 10 seconds with a Polytron (Westbury, New York). Mitochondrial and microsomal preparations were isolated by differential centrifugation.

Malondialdehyde quantitation. Each fraction was resuspended in KCl-tris buffer and incubated at 37C. One set of flasks was maintained on ice to serve as a 0 time control. Ferrous sulfate (1 mM.) was added to initiate lipid peroxidation. After 15, 30, 60, 90 or 120 minutes of incubation, respectively, an aliquot was removed from each flask and transferred to a microcentrifuge tube containing 40% trichloroacetic acid. Tubes were centrifuged at 1,000 g for 2 minutes. After centrifugation 100 μl. supernatant were incubated with 0.75 ml. 1% thiobarbituric acid, pH 7.4, at 90C for 30 minutes in a glass tube and then placed on ice for 10 minutes. At that point 2 ml. 1-butanol was added and each tube was capped and inverted for 2 minutes. Tubes were spun at 1,000 g for 5 minutes. Malondialdehyde formation was measured from the fluorescence intensity at an excitation wavelength of 532 nm. and an emission wavelength of 553 nm. using tetraethoxypropane as the standard. Quantitation of total protein was performed using Micro BCA Protein Assay (Pierce, Rockford, Illinois). Data were normalized and presented as nmol./ mg. protein malondialdehyde generated per 60 minutes of incubation.

α-Tocopherol. α-Tocopherol was extracted from mitochondrial and microsomal suspensions with hexane and the amount was quantified by high performance liquid chromatography using fluorescence detection with an excitation wavelength of 292 nm. and an emission wavelength of 340 nm. γ-Tocopherol was used as the internal standard. Statistical significance was determined by analysis of variance, followed by the Neuman-Keuls test with $p < 0.05$ considered statistically significant.

RESULTS

Figure 1 shows the α-tocopherol concentration in bladder muscle and mucosa after 4 and 6 weeks of the control and high α-tocopherol diets. The α-tocopherol concentrations were 5-fold greater in mitochondrial and microsomal preparations than in these preparations isolated from rabbits fed the normal diet. There were no differences in α-tocopherol concentrations at 4 and 6 weeks and, thus, for the experimental protocol we chose 4 weeks of feeding the supplemental diet before creating partial outlet obstruction.

Partial outlet obstruction resulted in a significant increase in bladder weight in obstructed groups. However, the increase in the rabbits fed the normal diet were significantly greater than in obstructed rabbits fed the high α-tocopherol diet (fig. 2).

Figure 3 shows the contractile responses to 4 and 32 Hz. field stimulation. The maximal tension generation to both frequencies of stimulation was reduced more than 60% in the

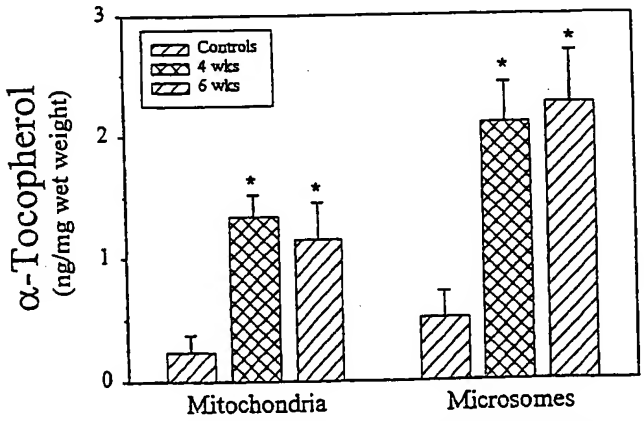

FIG. 1. α-Tocopherol concentration in bladder mitochondria and microsomes of 6 rabbits fed normal control diet containing 44 IU α-tocopherol per kg. and 6 fed normal diet supplemented with 1,000 IU/kg. α-tocopherol. Three rabbits in each group were evaluated at 4 and 6 weeks. Asterisk indicates significantly different from controls ($p < 0.05$).

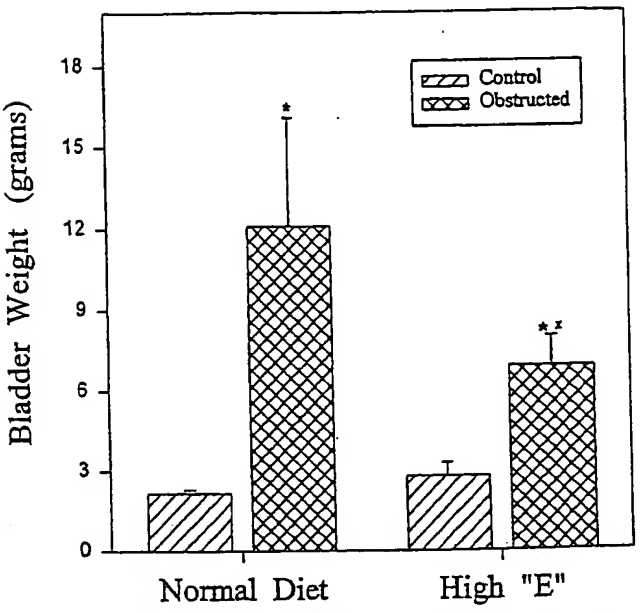

FIG. 2. Effect of α-tocopherol ("E") supplementation on bladder weight after partial outlet obstruction in 6 to 8 rabbits. Bars represent mean plus or minus standard of error of mean (SEM). Asterisk indicates significantly different from controls ($p < 0.05$). x, significantly different from normal diet ($p < 0.05$).

obstructed rabbits fed the normal diet, whereas the response in the obstructed group fed the high α-tocopherol diet was reduced between 10% and 20%. However, the maximal rate of tension generation was significantly reduced to the same extent in both obstructed groups. The responses to the other frequencies of stimulation were similar to those shown for 4 and 32 Hz. Thus, the high α-tocopherol diet resulted in significant protection of the maximal tension generation in response to field stimulation but not of the rate of tension generation.

Figure 4 shows the contractile responses to carbachol, KCl and adenosine triphosphate. For all 3 forms of stimulation the maximal contractile responses and maximal rate of tension generation were significantly reduced by partial outlet obstruction in the rabbits fed the normal diet, whereas no significant reductions in either parameter were noted in the obstructed rabbits fed the high α-tocopherol diet. Thus, feeding the rabbits a high α-tocopherol diet had a significant protective effect on the contractile responses to carbachol, KCl and adenosine triphosphate.

Figure 5 shows malondialdehyde in the microsomal and mitochondrial preparations. The malondialdehyde levels were 10 to 20-fold greater in the rabbits fed the normal diet than in the rabbits fed the high α-tocopherol diet in microsomal and mitochondrial preparations. Interestingly the malondialdehyde values in bladder smooth muscle of the obstructed bladders of the high α-tocopherol diet group were lower than the control high α-tocopherol diet group bladders.

Figure 6 shows α-tocopherol concentrations in the microsomal and mitochondrial preparations. α-Tocopherol concentrations in bladder muscle and mucosa from the high α-tocopherol diet group were significantly greater than in controls. Furthermore, concentrations in the mucosa were significantly greater than in smooth muscle. Interestingly the concentrations of α-tocopherol in the muscle and mucosa of obstructed rabbit bladders from the high α-tocopherol diet group were significantly higher than in control high α-tocopherol diet group bladders. This finding contrasts with those in the rabbits fed the normal diets, in which the concentrations of α-tocopherol were similar in the control and obstructed groups.

DISCUSSION

There is increasing evidence that cyclical ischemia and ischemia followed by reperfusion is an etiological factor for the progression of bladder dysfunction associated with experimental partial outlet obstruction.[5-9,12] During the ischemic period there is an increase in cytosolic free intracellular Ca^{+2} via release from the sarcoplasmic reticulum and mitochondria.[17-19] Increased free intracellular Ca^{+2} activates Ca^{+2}activated proteases such as calpain[20-22] and Ca^{+2} activated lipases such as phospholipase A_2,[21,23,24] which damage cell and subcellular organelle membranes. In addition, reperfusion (re-oxygenation) generates reactive oxygen species that cause lipid peroxidation of cellular membranes, further dysregulating Ca^{+2} homeostasis and perpetuating cell and subcellular organelle membrane damage.[17-19,25,26] A strong association between ultrastructural cellular and subcellular membrane damage, and the magnitude of contractile dysfunction was recently found in obstructed rabbit bladders and in men with obstructive dysfunction.[27-29]

Studies in an in vitro model of ischemia/re-oxygenation have demonstrated that the rate and magnitude of bladder contractile failure are directly related to the extracellular calcium concentration, which in turn is directly related to the level of lipid peroxidation after re-oxygenation.[13,14] The contractile function of the bladder strips could be protected by reducing the calcium concentration in the bath and by incubating the bladder tissues in the presence of calcium channel blockers.[30] These studies are consistent with studies using a rat model of partial outlet obstruction. The rat studies demonstrated that in vivo treatment with diltiazem (calcium channel blocker) significantly reduced the hypertrophic response of the bladder.[31]

Studies in the rabbit have demonstrated that treatment with Pygeum africanum before creating partial outlet obstruction significantly reduced the level of contractile and metabolic dysfunction induced by partial outlet obstruction.[32,33] In addition, treatment of rabbits that had previously undergone partial outlet obstructions resulted in a significant reversal of the contractile, biochemical and structural dysfunction induced by partial outlet obstruction.[34,35]

Although the mechanism of action of Pygeum africanum is not known, several components have significant antioxidant activity.[36] Our study demonstrated that feeding rabbits a diet high in α-tocopherol resulted in a 5-fold increase in α-tocopherol concentrations in the bladder membranes, substantially decreased baseline levels of the peroxidation product malondialdehyde and significantly improved contractile responses to all forms of stimulation. These results clearly show that a diet supplemented with α-tocopherol can have beneficial effects on bladders subjected to partial outlet obstruction.

Bladder outlet obstruction in men secondary to BPH may induce 2 sets of symptoms.[37] The obstructive symptoms (decreased voiding pressure, decreased flow rate and increased residual volume) relate directly to the decreased contractile responses of the bladder smooth muscle to field stimulation (nerve mediated contraction). However, the irritative symptoms (urgency, frequency and hyperreflexia) may relate to alterations in the sensory limb of the micturition reflex. The rabbit correlates of the obstructive symptoms directly relate to the decreased contractile responses to stimulation.[4,5,10] The rabbit correlates of the irritative symptoms (increased urinary frequency, decreased micturition volume and increased incidence of hyperreflexia) may relate to the increased mucosal permeability induced by obstruction and obstruction induced over distention and ischemia.[15,16]

Metabolic studies have demonstrated that the bladder mucosa is significantly more sensitive to anoxia and ischemia than bladder smooth muscle.[38,39] More recent studies have demonstrated that partial outlet obstruction induces a

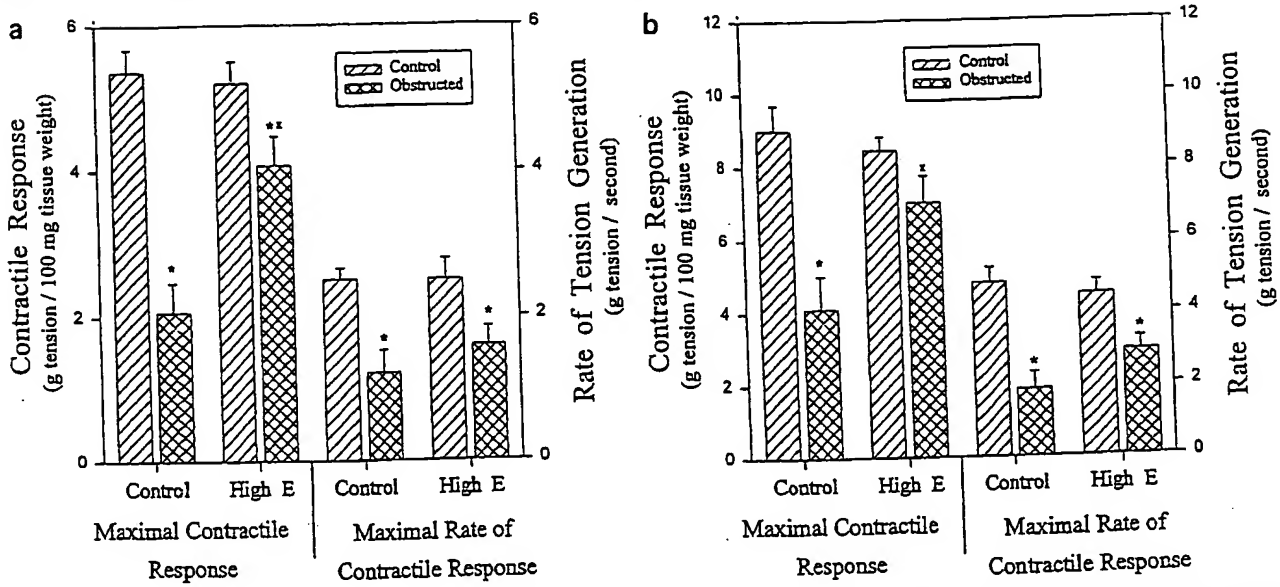

FIG. 3. Effect of α-tocopherol supplementation on contractile response to field stimulation after partial outlet obstruction in 6 to 8 rabbits. *a*, 4 Hz. stimulation. *E*, α-tocopherol. *b*, 32 Hz. stimulation. Bars represent mean plus or minus SEM. Asterisk indicates significantly different from controls ($p < 0.05$). *x*, significantly different from normal diet ($p < 0.05$).

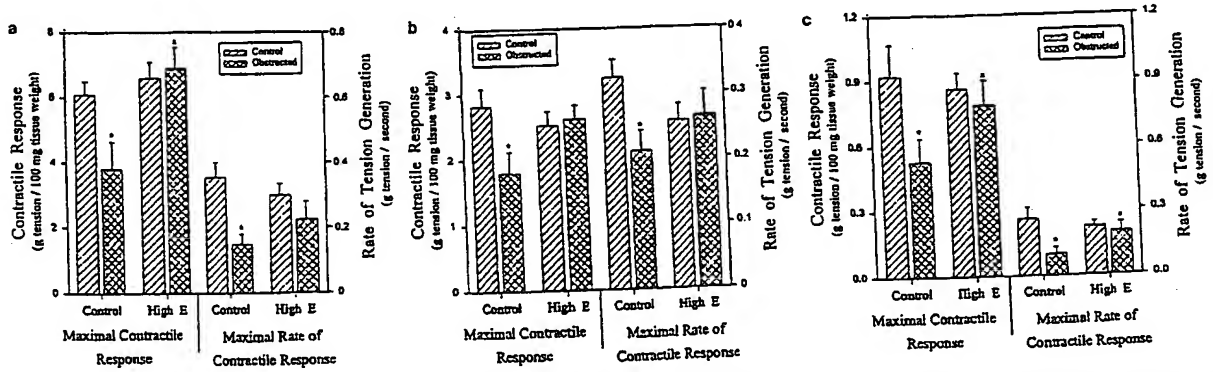

FIG. 4. Effect of α-tocopherol supplementation on contractile response after partial outlet obstruction in 6 to 8 rabbits. *a*, response to 20 mM. carbachol. *E*, α-tocopherol. *b*, response to KCl. *c*, response to 1 mM. adenosine triphosphate. Bars represent mean plus or minus SEM. Asterisk indicates significantly different from controls ($p < 0.05$).

marked increase in the membrane lipid metabolisms and activation of specific lipases, such as phospholipase A_2.[23,40] Future studies will be directed at increasing our understanding of the relationship of mucosal metabolism and the irritable symptoms of obstructive dysfunction.

Partial outlet obstruction resulted in a significantly greater increase in bladder mass in the untreated rabbits compared with the high α-tocopherol diet group. This observation is consistent with previous studies in rabbits and cats in which we have demonstrated that bladder weight is directly proportional to the level of contractile dysfunction.[10,41] That is, the increase in bladder mass is a protective response against the increasing residual volume as the contractile responses fail. The greater the contractile responses, the lower the bladder mass. Thus, the reduced rate of bladder hypertrophy (increase in mass) observed in the vitamin E treated rabbits was directly related to the reduced rate of contractile dysfunction in these rabbits.

It is interesting that, whereas vitamin E protected the bladder against the decrease in the maximal contractile responses to field stimulation, it did not protect against the decrease in the rate of contractile tension generation. This finding may be related to the marked calcium dysregulation that occurs after partial outlet obstruction.[4,10]

Pretreatment of the rabbits with vitamin E significantly reduced the level of malondialdehyde in the muscle and mucosa of control and obstructed rabbits. It is interesting that obstruction did not result in an increase in malondialdehyde in the muscle or mucosa and in fact after obstruction there was a reduced level of malondialdehyde in the smooth muscle of the vitamin E treated rabbits. Published studies have demonstrated that partial outlet obstruction results in significant hydrolysis of cellular and subcellular phospholipids, causing a significant increase in the release of free fatty acids.[23,40] We have noted that partial outlet obstruction results in a significant increase in lipid peroxidation for the first several days, which then is reduced to control levels by 1 and 2 weeks (unpublished data). We believe that increased cellular membrane phospholipid hydrolysis acts preferentially on the peroxidized lipids, thus, causing a net decrease in the level of peroxidation in the obstructed tissue. This hypothesis is currently being investigated.

One of the more interesting findings is that, although the concentrations of α-tocopherol were increased by feeding rabbits a diet high in α-tocopherol, concentrations in the obstructed bladders in the high α-tocopherol diet group were significantly greater than those in the control high α-tocopherol diet group. This finding may be related to the

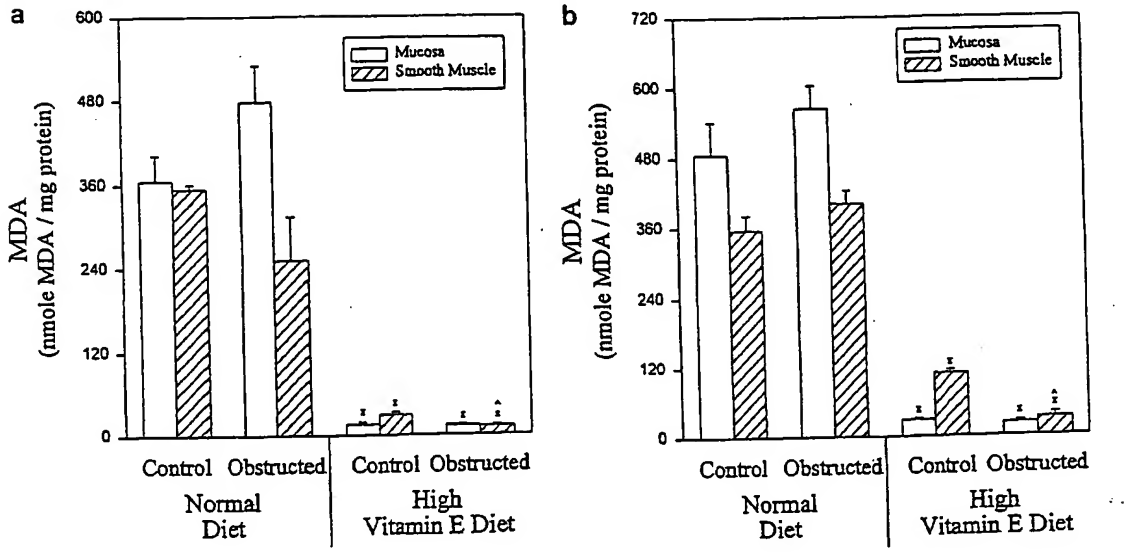

FIG. 5. Effect of α-tocopherol supplementation on Fe stimulation of malondialdehyde (*MDA*) after partial outlet obstruction in 6 to 8 rabbits. *a*, microsomal preparations. *b*, mitochondrial preparations. Bars represent mean plus or minus SEM. *x*, significantly different from normal diet ($p < 0.05$). ^, significantly different from controls ($p < 0.05$).

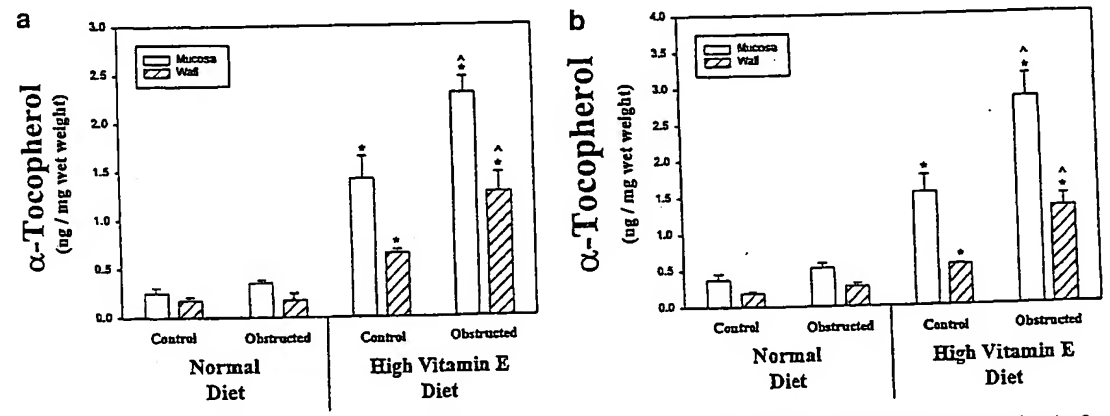

FIG. 6. Effect of α-tocopherol supplementation on α-tocopherol concentration in bladder after partial outlet obstruction in 6 to 8 rabbits. *a*, microsomal preparations. *b*, mitochondrial preparations. Bars represent mean plus or minus SEM. Asterisk indicates significantly different from normal diet ($p < 0.05$). ^, significantly different from controls ($p < 0.05$).

alterations in phospholipid metabolism observed after obstruction.[23,40]

The α-tocopherol levels correlate with the lower malondialdehyde levels in the smooth muscle of the obstructed high α-tocopherol diet group compared with the control high α-tocopherol diet group. It is a reasonable conclusion that the protective effect of α-tocopherol on bladder contractility is partially due to its increased tissue levels and antioxidant activity, as demonstrated by the reduced malondialdehyde concentrations. That the obstructed high α-tocopherol diet bladders had increased levels of α-tocopherol compared with the control high α-tocopherol diet bladders was an unexpected finding. However, the higher concentrations were consistent with the reduced levels of malondialdehyde in the obstructed high α-tocopherol diet bladders compared to the control high α-tocopherol diet bladders.

REFERENCES

1. Hinman, F., Jr. and Boyarsky, S.: Benign Prostatic Hypertrophy. New York: Springer-Verlag, 1983
2. Grayhack, J. T. and Kozlowski, J. M.: Benign prostatic hyperplasia. In: Adult and Pediatric Urology. Edited by J. Y. Gillenwater, J. T. Grayhack, S. S. Howards et al. Chicago: Year Book Medical, vol. 2, p. 1062, 1987
3. Zderic, S. A., Levin, R. M. and Wein, A. J.: Voiding function and dysfunction: relevant anatomy, physiology, pharmacology, and molecular aspects. In: Adult and Pediatric Urology, 3rd ed.. Edited by J. Y. Gillenwater, J. T. Grayhack, S. S. Howards et al. Chicago: Mosby Year Book, vol. 1, p. 1159, 1996
4. Levin, R. M., Longhurst, P. A., Monson, F. C. et al: Effect of bladder outlet obstruction on the morphology, physiology, and pharmacology of the bladder. Prostate, suppl., 3: 9, 1990
5. Levin, R. M., Brading, A. F., Mills, I. W. et al: Experimental models of bladder obstruction. In: Prostatic Disease. Edited by H. Lepor. Philadelphia: W. B. Saunders, chapt. 13, p. 169, 1999
6. Greenland, J. E. and Brading, A. F.: Urinary bladder blood flow changes during the micturition cycle in a conscious pig model. J Urol, 156: 1858, 1996
7. Azadzoi, K. M., Pontari, M., Vlachiotis, J. et al: Canine bladder blood flow and oxygenation: changes induced by filling, contraction and outlet obstruction. J Urol, 155: 1459, 1996
8. Lin, A. T., Chen, M. T., Yang, C. H. et al: Blood flow of the urinary bladder: effects of outlet obstruction and correlation with bioenergetic metabolism. Neurourol Urodyn, 14: 285, 1995

9. Brading, A. F.: Alterations in the physiological properties of urinary bladder smooth muscle caused by bladder emptying against an obstruction. Scand J Urol Nephrol, suppl., 184: 51, 1997
10. Kato, K., Monson, F. C., Longhurst, P. A. et al: The functional effects of longterm outlet obstruction on the rabbit urinary bladder. J Urol, 143: 600, 1990
11. Nigro, D. A., Haugaard, N., Wein, A. J. et al: Cellular basis for contractile dysfunction following chronic partial bladder outlet obstruction in rabbits. Mol Cell Biochem, 200: 1, 1999
12. Greenland, J. E., Hvistendahl, J. J., Andersen, H. et al: Detrusor-and kidney blood flow is reduced in response to early bladder outlet obstruction in pigs. J Urol, suppl., 157: 172, abstract 666, 1997
13. Levin, R. M., Leggett, R., Whitbeck, C. et al: Effect of calcium and calcium chelators on the response of the bladder to in vitro ischemia. Br J Urol, 82: 882, 1998
14. Ohnishi, N., Liu, S.-P, Horan, P. et al: Effect of repetitive stimulation on the contractile response of rabbit urinary bladder subjected to in vitro hypoxia or in vitro ischemia followed by reoxygenation. Pharmacology, 57: 139, 1998
15. Levin, R. M., Hypolite, J. A., Haugaard, N. et al: Comparative response of rabbit bladder smooth muscle and mucosa to anoxia. Neurourol Urodyn, 15: 79, 1996
16. Brigelius-Flohe, R. and Traber, M. G.: Vitamin E: function and metabolism. FASEB J, 13: 1145, 1999
17. Richter, C. and Kass, G. E.: Oxidative stress in mitochondria: its relationship to cellular Ca2+ homeostasis, cell death, proliferation, and differentiation. Chem Biol Interact, 77: 1, 1991
18. Richter, C., Gogvadze, V., Laffranchi, R. et al: Oxidants in mitochondria: from physiology to diseases. Biochim Biophys Acta, 1271: 67, 1995
19. Cassarino, D. S. and Bennett, J. P., Jr.: An evaluation of the role of mitochondria in neurodegenerative diseases: mitochondrial mutations and oxidative pathology, protective nuclear responses, and cell death in neurodegeneration. Brain Res Rev, 29: 1, 1999
20. Zhao, Y., Levin, S. S., Wein, A. J. et al: Correlation of ischemia/reperfusion and partial outlet obstruction induced spectrin proteolysis by calpain with contractile dysfunction in the rabbit bladder. Urology, 49: 293, 1997
21. Trump, B. F. and Berezesky, I. K.: Calcium-mediated cell injury and cell death. FASEB J, 9: 219, 1995
22. Saido, T. C., Sorimachi, H. and Suzuki, K.: Calpain: new perspectives in molecular diversity and physiological-pathological involvement. FASEB J, 8: 814, 1994
23. Hass, M. A., Leonova, E. and Levin, R. M.: Fatty acid profiles in normal and obstructed rabbit bladder smooth muscle and mucosa. Neurourol Urodyn, 18: 697,1999
24. Verity, M. A.: Mechanisms of phospholipase A2 activation and neuronal injury. Ann N Y Acad Sci, 679: 110, 1993
25. Lin, A. T.-L., Yang, C. H., Chang, K. K. et al: Oxygen free radicals induced lipid peroxidation in overdistention of the rabbit urinary bladders. Neurourol Urodyn, suppl., 14: 553, 1995
26. Lin, A. T.-L., Yang, C. H., Chen, K. K. et al: Correlation of lipid peroxidation with energetic metabolism and contractile function in overdistention of rabbit urinary bladder. Neurourol Urodyn, suppl., 15: 426, 1996
27. Gosling, J. A., Kung, L. S., Dixon, J. S. et al: Correlation between the structure and function of the rabbit urinary bladder following partial outlet obstruction. J Urol, 163: 1349, 2000
28. Lu, S.-H., Wei, Y.-H., Chang, L. S. et al: Morphological and morphometric analysis of human detrusor mitochondria with urodynamic correlation after partial bladder outlet obstruction. J Urol, 163: 225, 2000
29. Levin, R. M., Haugaard, N., O'Connor, L. et al: Obstructive response of human bladder to BPH vs. rabbit bladder response to partial outlet obstruction: a direct comparison. Neurourol Urodyn, 19: 609, 2000
30. Levin, R. M, Leggett, R. E., Whitbeck, C. et al: Effect of diltiazem and pinacidil on the response of the rabbit urinary bladder to repetitive stimulation and in vitro ischemia. Neurourol Urodyn, 18: 129, 1999
31. Steers, W. D., Albo, M. and Tuttle, J. B., Calcium channel antagonists prevent urinary bladder growth and neuroplasticity following mechanical stress. Am J Physiol, 266: R20, 1994
32. Levin, R. M., Riffaud, J.-P., Bellamy, F. et al: Protective effect of Tadenan on bladder function secondary to partial outlet obstruction. J Urol, 155: 1466, 1996
33. Levin, R. M., Riffaud, J.-P., Bellamy, F. et al: Effects of Tadenan pretreatment on bladder physiology and biochemistry following partial outlet obstruction. J Urol, 156: 2084, 1996
34. Levin, R. M., Das, A. K., Haugaard, N. et al: Beneficial effects of Tadenan therapy following two weeks of partial outlet obstruction in the rabbit. Neurourol Urodyn, 16: 583, 1997
35. Gomes, C. M., DiSanto, M., Horan, P. et al: Improved contractility of obstructed bladders after Tadenan treatment is associated with reversal of altered myosin isoform expression. J Urol, 163: 2008, 2000
36. Hass, M. A., Nowak, D. M., Leonova, E. et al: Identification of components of Prunus africana extract that inhibit lipid peroxidation. Phytomedicine, 6: 379, 1999
37. Jepsen, J. V. and Bruskewitz, R. C.: Clinical manifestations and indications for treatment. In: Prostatic Diseases. Edited by H. Lepor. Philadelphia: W. B. Saunders, chapt. 10, p. 127, 1999
38. Hypolite, J. A., Longhurst, P. A., Gong, C. et al: Metabolic studies on rabbit bladder smooth muscle and mucosa. Mol Cell Biochem, 125: 35, 1993
39. Levin, R. M., Hypolite, J. A., Haugaard, N. et al: Comparative response of rabbit bladder smooth muscle and mucosa to anoxia. Neurorurol Urodyn, 15: 79, 1996
40. O'Connor, L. J., Goldner, C. W., Lau, S. T. et al: Effect of partial outflow obstruction on the distribution of free fatty acids and phospholipids in the rabbit bladder. World J Urol, 17: 261, 1999
41. Levin, R. M., Longhurst, P. A., Barasha, B. et al: Studies on experimental bladder outlet obstruction in the cat: long-term functional effects. J Urol, 148: 939, 1992

Acta Neurol Scand 1998: 98: 145–153
Printed in UK – all rights reserved

ACTA NEUROLOGICA SCANDINAVICA
ISSN 0001-6314

Review article

Oxidative injury in the nervous system

Delanty N, Dichter MA. Oxidative injury in the nervous system. Acta Neurol Scand 1998: 98: 145–153. © Munksgaard 1998.

N. Delanty, M. A. Dichter

Department of Neurology, Hospital of the University of Pennsylvania, Philadelphia, PA 19104-4283, USA

A free radical is a highly reactive chemical species that can react with organic macromolecules leading to cell and tissue damage and consequent functional disruption. Free radical or oxidative injury is increasingly recognized as an important factor in the pathophysiology of many human diseases, including those that affect the nervous system. This review summarizes important evidence implicating oxidative injury in the pathogenesis and progression of many important neurological disorders, including cerebrovascular disease, epilepsy, amyotrophic lateral sclerosis, and Huntington's disease. Results of controlled clinical trials of various antioxidant therapies in neurological disease performed to date are also highlighted.

Key words: oxidative injury; free radical injury; nervous system; neurological disease

N. Delanty, Dept of Neurology, Hospital of the University of Pennsylvania, 3, West Gates, 3400 Spruce Street, Philadelphia, PA 19104-4283, USA

Accepted for publication May 12, 1998

Oxidative or free radical injury is a fundamental mechanism of human disease (1, 2). Increasing evidence suggests that such injury is important in the pathogenesis of a diverse group of neurological disorders (3), such as cerebrovascular disease, mitochondrial disorders, amyotrophic lateral sclerosis, Huntington's disease, Alzheimer's disease, and epilepsy. Elucidation of the role of oxidative injury is important because therapy with agents that scavenge free radicals and augment endogenous antioxidant capacity may be useful in the therapeutic modulation of these devastating neurological conditions. This review outlines the potential importance of free radical injury in the nervous system and highlights new information and clinical trial data in this expanding area.

A free radical is an atom or molecule with an unpaired electron in its outer orbit. This state makes it highly reactive with other chemical species. The biochemistry of organic oxidative injury is complex (Fig. 1) (4, 5). The superoxide anion (O_2^-) is formed as a byproduct of normal metabolism. Superoxide undergoes spontaneous dismutation to form hydrogen peroxide, a reaction which is catalysed by superoxide dismutase. Hydrogen peroxide is subsequently removed by the action of catalase. Peroxynitrite is formed by the reaction between superoxide anion and nitric oxide, and it and its breakdown products (the nitronium anion and the hydroxyl radical) are highly toxic to the nervous system. Iron and other metal ions favour the formation of the highly reactive hydroxyl radical.

Free radicals "attack" important macromolecules leading to cell damage and homeostatic disruption. Free radicals can react with the lipid bilayer of cell membranes and alter their membrane fluidity characteristics, and also lead to release of potentially toxic byproducts. Free radicals react with proteins leading to enzyme inactivation and disruption of cellular function. They also react with DNA and RNA leading to somatic mutations, and to disturbances of transcription and translation.

Other fundamental processes involved in the pathogenesis of neuronal damage and disease appear to involve free radical-mediated injury. For example, glutamate neurotoxicity is mediated at least in part by the liberation of free radicals. Cortical neurons from transgenic mice overexpressing copper-zinc-superoxide dismutase are protected *in vitro* against glutamate induced neuronal damage (6). Free radicals may also be important in the induction or mediation of programmed cell death or apoptosis (7, 8), and in signal transduction and gene expression (9).

Endogenous antioxidant mechanisms exist to protect against the oxidative injury associated with normal metabolism (10). Mechanisms which protect cells against oxidative injury include compartmentalization of cellular processes, e.g. in peroxisomes; enzyme systems such as glutathione peroxidase, superoxide dismutase, and catalase; specific transport proteins and binding molecules for iron and other metals; and endogenous antioxi-

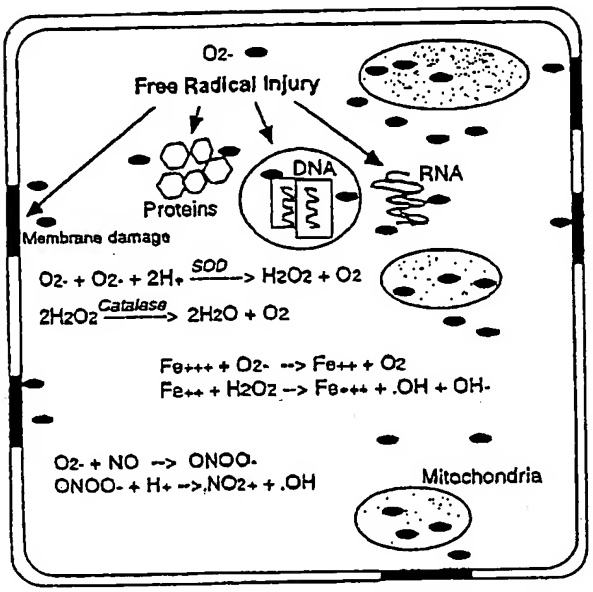

Fig. 1. Cell diagram depicting important free radical reactions. The superoxide anion ($O_2^{\cdot-}$, small black oval symbol) is formed as a result of normal metabolism, and subsequently converted to hydrogen peroxide (H_2O_2) and then to water and oxygen by the action of the enzymes superoxide dismutase and catalase respectively. The presence of transition metal ions such as iron favours the formation of the hydroxyl radical ($\cdot OH$), a potent pro-oxidant. Peroxynitrite ($ONOO^-$), and nitronium ($\cdot NO_2^+$), as well as the hydroxyl radical are formed by the reaction of superoxide anion and nitric oxide (NO), and these further contribute to oxidant stress. Increased free radical production or decreased inactivation may lead to disease by oxidative damage to proteins, lipid membranes, and/or nucleic acids.

dant compounds which can abort free radical chain reactions such as water soluble ascorbic acid (vitamin C) and lipid soluble α-tocopherol (vitamin E). The molecular basis of some human diseases may involve abnormalities of these endogenous defense systems. The process of aging may in part be due to the gradual overwhelming of these protective endogenous antioxidant defenses by chronic metabolic stress, a sort of "aging metabolic clock" (11, 12). In support of this, mitochondrial genotype has recently been suggested to be associated with longevity (13). In addition, increased age is clearly a risk factor in many diseases which are associated with oxidative injury, such as atherosclerosis, Alzheimer's disease, and cancer.

In some circumstances, increased local release of free radicals may be beneficial. For example, release of toxic oxidant species by activated neutrophils may play a role in host defense against infection. Similarly, the local release of free radicals by immunocompetent cells may be important in surveillance against early cancer. Much like inflammation, oxidant reactions can be both detrimental and beneficial to the organism.

Measurement of oxidative injury

Free radicals are evanescent chemical species. Thus, the elucidation of the importance of oxidative injury in human disease in general has been hampered by the difficulty in directly measuring free radicals. Measurement *in vivo* has relied on indirect means of assessing free radical injury such as measurement of malondialdehyde, conjugated dienes, or breath pentane, or *ex vivo* by electron paramagnetic resonance. However, the reliability of such methods, their sensitivity and specificity, and their application to what actually happens *in vivo* in terms of free radical biology has been questioned (14, 15).

Over the last few years novel methods of assessing oxidant status in human disease have been developed which permit more accurate assessment of the importance of free radical injury in disease, and which may also provide additional methods of defining optimal doses of putative antioxidants in therapy. Such methods include the measurement of 3-nitrotyrosine, a stable by-product of peroxynitrite (16); protein carbonyl derivatives, markers of protein oxidation (17); isoprostanes, stable products of arachidonic acid formed under conditions of oxidative stress in a cyclo-oxygenase independent manner (18, 19); and 8-hydroxy-2-deoxyguanisine, a stable byproduct of DNA oxidant injury (20).

Neurological diseases associated with oxidative injury

It is difficult to find a human disease in which a study implicating oxidative injury does not exist. Despite this, there is still uncertainty and controversy as to whether oxidative injury is a cause or consequence of disease (21), or whether appropriate therapy with effective free radical scavengers can prevent disease or favorably modulate its progression (22, 23). It is likely that the truth of the "cause or consequence" argument lies somewhere in the middle – some diseases are likely to be caused by perturbations in factors which regulate free radical homeostasis, and other diseases may initially be independent of such factors but the disorder itself leads to an increase in oxidative stress which may further exacerbate tissue injury and disease progression. Effective therapy may be of critical importance in either case. Despite the extensive basic neuroscience research that implicates oxidative injury in neurological diseases, there have been few well conducted prospective controlled studies of putative antioxidant compounds in the modulation of these disorders. Important published trials performed to date are summarized in Table 1. None of these trials have

Table 1. Summary of selected randomized placebo-controlled trials of putative antioxidants in neurological disorders

Disorder	Trial (ref)	Agent	n (treated)	n (placebo)	Result
Stroke (<6 h)	34	Tirilazad 6 mg/kg/day×3 days	276	280	Neg.
SAH (<48 h)	44[1]	Tirilazad 6 mg/kg/day×8–10 days	256	253	Pos, $P<0.01$
SAH (<48 h)	45	Tirilazad 6 mg/kg/day×8–10 days	299	300	Neg.
Alzheimer's (moderate)	48	Vitamin E 2,000 IU/day×2 years	170	171	Pos, $P<0.001$
Alzheimer's (mild–severe)	50	Ginkgo biloba (extract) 120 mg/day×1 year	75	75	Pos, $P<0.005$
ALS	61	Acetylcysteine 50 mg/kg/day×12 mths	55	56	Neg.
Epilepsy (children)	71	Vitamin E 400 IU/day×3 mths	12	12	Pos, $P<0.05$
Parkinson's (early)	81	Vitamin E 2000 IU/day×14±6 mths	399	401	Neg.
HD (mild–moderate)	84[2]	Vitamin E 3000 IU daily×1 year	40	33	Neg
HD (mild–moderate)	85	Idebenone 90 mg tid×1 year	48	43	Neg.
SC injury (within 8 h)	89[3]	Tirilazad 2.5 mg/kg q 6 h×48 h	150	145 (149)	Pos.

See text and individual references for discussion. SAH: sub-arachnoid hemorrhage; ALS: amyotrophic lateral sclerosis; HD: Huntington's disease.

[1] In two other arms of this study patients treated with either 0.6 mg/kg/day or 2 mg/kg/day had no better outcome when compared to those given vehicle alone.

[2] Both arms of this study were given vitamin A 25,000 IU twice daily and vitamin C 500 mg twice daily.

[3] Forty eight hours of tirilazad treatment was equivalent at 6 months to 24 h of methylprednisolone treatment (145 patients) at a dose that has been previously been shown to be superior to placebo; but was not as effective as 48 h of methylprednisolone treatment (149 patients).

attempted to measure biological indices of free radical injury as a surrogate marker for drug efficacy.

Cerebrovascular disease

The importance of oxidant injury in atherogenesis has been extensively studied in the last two decades. The facilitated uptake of oxidized LDL by macrophages to form foam cells is a crucial early event in atherosclerosis (24). Although the biology of free radical injury in vascular disease has been most extensively studied in the coronary circulation, a number of observations indicate that oxidative injury is also of fundamental importance in the pre-cerebral and cerebral vasculature. Firstly, antibodies against oxidized LDL have been detected in human carotid plaque and correlate with disease severity (25). Secondly, increased levels of isoprostanes, oxidative non-enzymatic derivatives of arachidonic acid, have been found in human atherosclerotic plaque isolated from carotid endarterectomy specimens when compared to vascular tissue devoid of atherosclerosis (26). In addition, immunohistochemical studies confirmed that foam cells within the plaque were strongly positive for 8-epi-prostaglandin F_{2a}, an abundant isoprostane. Thirdly, a significant increase in serum markers of cholesterol oxidation and of LDL-thiobarbituric acid-reacting substances (TBARS) has been demonstrated in patients with rapid progression of carotid atherosclerosis compared to subjects with little or no progression of disease as measured by high resolution B-mode ultrasonography (27).

Epidemiological studies have shown that the intake of antioxidant vitamins correlate inversely with carotid plaque thickness (28); and observational studies have shown a positive association between plasma TBARS and presence of carotid plaque in elderly men (29), and an inverse association between erythrocyte vitamin E levels and common carotid artery intima-media thickness in both sexes in the same study. Thus basic and epidemiological data support the role of free radical injury in carotid atherosclerosis, although to our knowledge, no published prospective studies of the effect of free radical scavengers on ultrasonically-assessed progression of carotid atherosclerosis exist. Vitamin E supplementation in the secondary prevention of ischaemic stroke is currently being examined in patients with established vascular disease in the British Heart Foundation trial (30). Dietary intake of flavonoids (mainly quercetin found in tea, fruit, and vegetables) has been pro-

spectively associated with a reduced incidence of stroke (31).

Free radicals may also play a role in the cascade of events which lead to cellular injury following ischaemia (32), and the generation of free radicals may potentiate glutamate neurotoxicity in this situation (6). Increased urinary excretion of 8-epi-prostaglandin $F_{2\alpha}$, has been demonstrated in the first 12 to 24 h following acute ischaemic stroke when compared to age matched controls (33). It is plausible that effective antioxidants could save the ischaemic penumbra from cell death and thus limit neurological dysfunction after stroke. The only trial of a putative antioxidant in acute stroke to date has not shown benefit. Tirilazad, a 21-aminosteroid inhibitor of lipid peroxidation, given to 276 patients within 6 h (median, 4.3 h) of stroke onset had no beneficial effect at 3 months when compared with 280 patients given placebo (34) (Table 1).

Increased oxidative stress has been demonstrated in chronic cigarette smoking (35), hypertension (36), and hypercholesterolaemia (37). The impact of these potent risk factors for vascular disease and stroke could potentially be ameliorated by chronic antioxidant supplementation.

Reperfusion injury may be associated with the generation of free radicals (38). Evidence for this is most convincing in the coronary circulation following thrombolytic therapy or primary angioplasty for acute myocardial infarction, or after global myocardial reperfusion that occurs following coronary bypass surgery (39, 40). It is unknown whether oxidative reperfusion injury occurs in the brain following treatment of ischaemic stroke with tissue plasminogen activator (tPA), thus possibly attenuating the effectiveness of this therapy. Clinical deterioration following initial improvement without hemorrhage or increased oedema is known to occur, and may be a manifestation of this phenomenon. If this is the case, adjuvant therapy with free radical scavengers used with tPA may prove useful.

Cerebral vasospasm is a major cause of morbidity and mortality following subarachnoid haemorrhage (SAH). Evidence suggests that this may be mediated by production of free radical species (41). The release of biologically active isoprostanes formed during lipid peroxidation has been shown to cause cerebral vasospasm in an animal model (42). Recently, increased plasma levels of cholesterol ester hydroperoxides (markers of free radical injury) correlated with increased mortality in patients with SAH (43). In addition, in the same study, plasma ascorbic acid levels were inversely correlated with the risk of vasospasm. Two large clinical trials using tirilazad 6 mg/kg/day given for 8 to 10 days following onset of SAH have yielded conflicting results (44, 45) (Table 1), and use of higher doses of tirilazad are ongoing (45).

Alzheimer's disease

Evidence that suggests that oxidative stress is important in the neurodegeneration of Alzheimer's disease (AD) has recently been extensively reviewed (46). Increased lipid peroxidation, protein and DNA oxidation, and formation of advanced glycation end-products have all been reported in patients with AD (47). Use of high dose vitamin E 2000 IU per day for 2 years has recently been shown to reduce the incidence of death, institutionalization, loss of activities of daily living, and severe dementia in patients with moderate disease (48). Additionally, the antioxidant flavonoids have been shown to be neuroprotective *in vitro* (49), and a controlled trial using a plant extract of *Ginkgo biloba*, which contains flavonoids and other antioxidants, has shown benefit in Alzheimer's disease. In this study, there was a significantly better outcome in terms of Alzheimer's Disease Assessment Scale – Cognitive subscale (ADAS-Cog), and Geriatric Evaluation by Relative's Rating Instrument in patients with mild to moderately severe dementia after 1 year's treatment when compared to placebo, without increase in adverse effects (50). In this trial there was no difference in Clinical Global Impression of Change, a measure of the physician's judgement of overall change in impairment compared to baseline. However, it is remarkable that this short trial demonstrated objective improvement (by ADAS-Cog), and subjective improvement as judged by the patient's blinded caregiver.

In addition to Alzheimer's disease, oxidative injury to neurons may be important in other forms of dementia. In corroboration of this, a novel scavenger of superoxide anion and a structural homologue of vitamin E, OPC-14117, has recently shown promise in a small pilot trial in patients with HIV-associated cognitive impairment (51).

Amyotrophic lateral sclerosis

The landmark discovery that some familial forms of amyotrophic lateral sclerosis (ALS) are caused by autosomal dominant mutations in the gene encoding for cytosolic copper–zinc superoxide dismutase (52) has led to enormous interest in potential oxidative mechanisms involved in the pathogenesis of both familial and non-familial ALS. The mechanism by which a mutation in superoxide dismutase (SOD) could lead to neuronal cell death is unclear, as SOD knockout mice fail to develop an animal model of the disease (53).

Moreover, it is unlikely that the capacity of catalase to inactivate the increased formation of hydrogen peroxide is overwhelmed, as transgenic mice overexpressing the human wildtype SOD gene also fail to develop the animal model of the disease (54).

The most popular hypothesis explaining familial ALS is that the mutated enzyme leads to enhanced neurotoxicity which in some way damages the neuron – so called "gain of function" (55). Transgenic mice overexpressing some mutated forms of the SOD gene causing human familial ALS go on to develop disease (54). It has been speculated that the mutated forms of SOD somehow enhances formation of peroxynitrite within the nervous system (55). Vitamin E supplementation delays onset of clinical disease in the transgenic mouse model of familial ALS, but does not prolong survival (56).

Data from humans support the role of oxidative injury in non-familial ALS. Autopsy studies have shown increased protein carbonyl levels in the spinal cords of patients in comparison to controls (57). Glutathione peroxidase, but not SOD, activity was reduced in the precentral gyrus of autopsy specimens from 9 patients with sporadic ALS in comparison to 9 patients dying of non-neurological causes (58). Increased nitrotyrosine, a marker for peroxynitrite production, has also been shown to be elevated in post-mortem studies in both sporadic and familial ALS (59), and elevated nitrotyrosine immunoreactivity has been found in the cerebrospinal fluid of patients with ALS (60). In patients with ALS, a 1-year clinical trial of *N*-acetylcysteine, an antioxidant and glutathione precursor, failed to show any benefit, although there was a non-significant trend in favour of the active treatment group (61). Recently a mitochondrial cytochrome *c* oxidase subunit deletion has been found in a patient with early onset motor neuron disease (62). Thus, defects in oxidative phosphorylation leading to increased oxidant stress may underlie some human phenotypes of ALS.

Epilepsy

Seizures are the symptoms of epilepsy, a disorder which is being increasingly recognized as a chronic dynamic neurological disorder (63). The focus of antiepileptic drug therapy may gradually change from seizure control to modulation of the underlying disease process. Data exist to suggest that oxidative injury may be important in epilepsy, although information in humans is still preliminary. Reports from studies in animals suggest that free radical injury is involved in the pathogenesis of post-traumatic epilepsy. In rats, injection of iron salts into the cortex lead to the formation of superoxide radicals (64), and in a model of iron-induced seizures, adenosine scavenged hydroxyl radicals and prevented post-traumatic epilepsy (65). In another study in rats, epileptiform activity measured by electrocorticography correlated with increased lipid peroxidation (66), and a related study showed that α-tocopherol and selenium protected against iron-induced epileptiform activity and gliosis (67). Oxidative stress induced apoptotic cell death in embryonic cortical neurons *in vitro* (68), and was prevented by the antioxidant *N*-acetylcysteine (69). The 21-aminosteroid, U-74389G, an inhibitor of lipid peroxidation, prevented hippocampal damage in a rat model of dendrotoxin-K induced seizures (70). Oxidative stress may change channel permeability and thus modulate epileptogenesis. The results of a small prospective randomized trial in children with intractable epilepsy suggested that vitamin E 400 IU per day for 3 months significantly improved seizure control (71). However, the complete absence of a placebo response in this trial was unusual. Also in patients, *N*-acetylcysteine delayed the progression of the progressive myoclonic epilepsy syndrome, Unverricht–Lundborg disease (72). Deficiency of selenium, a cofactor in several endogenous antioxidant enzymes, has been suggested to play a causative role in two children with intractable seizures (73). Finally, there is also evidence that treatment with anticonvulsants can cause or exacerbate free radical injury (74).

Parkinson's disease

The nigrostriatal degeneration which underlies the expression of Parkinson's disease may be due to mechanisms that involve free radical neurotoxicity. Evidence exists to suggest that oxidative injury is important in the dopaminergic cell death in this disorder. Excessive accumulation of iron has been described in the pars compacta of the substantia nigra in patients with Parkinson's disease (75). Reduced glutathione, an important endogenous tripeptide which removes hydrogen peroxide, is significantly reduced in the substantia nigra of post-mortem brains of patients with Parkinson's disease (76). The MPTP-induced form of Parkinson's disease is also associated with increased oxidative stress (77). Importantly, therapy with L-dopa may lead to the generation of oxy-radicals (78), and it is unclear whether such therapy contributes to neuronal damage and accelerates disease progression. This has obvious implications for the timing of initiation of L-dopa therapy, a controversial issue which remains to be resolved.

Dopaminergic cell transplantation may have a role in the future therapy of Parkinson's disease. Such therapy is in part hampered by the low

survival rate of transplanted neurons. Lazaroids or 21-aminosteroids have been shown to ameliorate cell loss in a rat model of transplanted dopamine neurons (79). Furthermore, survival of dopaminergic neurons was four times greater when harvested from transgenic mice overexpressing copper–zinc SOD when compared to those taken from normal mice (80). Thus oxidative damage to dopamine neurons may be an important factor in limiting transplant efficacy, and may be ameliorated by antioxidants.

The only randomized prospective controlled trial of antioxidant therapy in Parkinson's disease failed to show any benefit from supplementation with vitamin E 2000 IU daily for a mean period of 14 months (81). Vitamin E is mainly effective in aborting oxidative chain reactions within the lipid-rich bilayer of the cell membrane, and thus may not be an appropriate antioxidant to treat Parkinson's disease. In addition, the optimal scavenging capacity of vitamin E may require coadministration of high dose vitamin C (82). It is possible that therapy with other oxidant scavengers, particularly those that are most effective within the cytosol, those that sequester iron, or those that augment brain glutathione may be beneficial.

Huntington's disease

In parallel to the nigostriatal dopaminergic neurodegeneration seen in Parkinson's disease, the striatal pathology of Huntington's disease may also be due in part to oxidant injury. Glutamate neurotoxicity in the basal ganglia in Huntington's disease may be mediated by the release of intracellular free radicals, and thus ameliorated by antioxidants. However, the exact role of the abnormal accumulation of the mutated protein, huntingtin, and its relationship to glutamate and oxidative neurotoxicity is presently unknown. In support of the role of oxidative injury in Huntington's disease, increased levels of 8-hydroxydeoxyguanosine have been found in caudate nuclear DNA in postmortem brain tissue of 18 patients compared to that of 29 controls without neurological disease (83). In the same study, no difference in caudate cytosolic SOD activity was observed between patients and controls.

Two small controlled trials of antioxidant therapy in patients with mild to moderate Huntington's disease have been performed (Table 1). Vitamin E (3000 IU daily for 1 year) had no overall effect on neurological or neuropsychiatric symptoms, although *post hoc* analysis suggested a beneficial effect in those with early disease (84). In addition, both the placebo and the active treatment group were given additional antioxidant treatment with high-dose vitamin C and vitamin A. This may have diluted any potential benefit of vitamin E. In another study by the same group, treatment for 1 year with 270 mg per day of idebenone, a benzoquinone antioxidant, failed to yield benefit (85).

Spinocerebellar ataxia

Perturbations in vitamin E metabolism have been associated with spinocerebellar dysfunction presenting with a clinical phenotype similar to Friedrich's ataxia. Bassen–Kornzweig disease is an inborn deficiency of very low density lipoproteins which normally transport lipid soluble vitamin E from the gastrointestinal system to the nervous system. Treatment with high dose vitamin supplementation can be effective in reversing neurological dysfunction. Mutations in the α-tocopherol transport protein can also lead to similar spinocerebellar disturbance, and treatment with vitamin E is effective (86). Similar mutations in the α-tocopherol transport protein are also associated with retinitis pigmentosa (87). Thus patients with unexplained spinocerebellar ataxia, especially when associated with retinitis pigmentosa, should be given a trial of high-dose α-tocopherol.

Head and spinal cord injury

Traumatic injury to the nervous system is a dynamic process which involves lipid peroxidation and other harmful oxidative events which occur subsequent to the initial insult (88). Early treatment with radical scavengers may help in salvaging some of the compromised tissue analogous to attempts to protect the ischaemic penumbra following acute vascular occlusion. In acute spinal cord injury, treatment with 48 h of tirilazad has been shown to have similar efficacy to 24 h of high dose methylprednisolone, which has previously been shown to be superior to placebo (89). A controlled trial of tirilazad following acute head injury has been completed and published results are awaited (90).

Conclusions

Much evidence suggests that oxidative mechanisms are important in cell injury in many neurological diseases. Clinical trials of putative antioxidants have been performed in recent years with mixed results. Newer techniques of measuring free radical damage in human disease are allowing for a better understanding of oxidative injury in the nervous system. In addition, these techniques could be used in dose finding studies of antioxidant effectiveness before expensive large scale clinical trials are performed. Advances in neurogenetics and pre-dis-

ease screening, as well as new knowledge about the progressive nature of the neuropathology underlying a number of important neurological diseases, compel us to consider the option of treating at risk individuals with agents such as antioxidants that may delay or ameliorate some of these disorders.

References

1. Cross CE, moderator. Oxygen radicals and human disease. Ann Intern Med 1987: 107: 526–45.
2. McCord JM. Human disease, free radicals, and the oxidant/antioxidant balance. Clin Biochem 1993: 26: 351–7.
3. Jenner P. Oxidative damage in neurodegenerative disease. Lancet 1994; 344: 796–8.
4. Cohen G. Enzymatic/nonenzymatic sources of oxyradicals and regulation of antioxidant defenses. Ann NY Acad Sci 1994: 738: 8–14.
5. Villa RF, Gorini A. Pharmacology of lazaroids and brain energy metabolism: a review. Pharm Rev 1997: 49: 99–136.
6. Chan PH, Chu L, Chen SF, Carlson EJ, Epstein CJ. Reduced neurotoxicity in transgenic mice overexpressing human copper-zinc-superoxide dismutase. Stroke 1990: 21(Suppl. 111): 80–2.
7. Greenlund LJS, Deckwerth TL, Johnson EM. Superoxide dismutase delays neuronal apoptosis: a role of reactive oxygen species in programmed neuronal death. Neuron 1995: 14: 303–15.
8. Stoian I, Oros A, Moldoveanu E. Apoptosis and free radicals. Biochem Mol Med 1996: 59: 93–7.
9. Palmer HJ, Paulson KE. Reactive oxygen species and antioxidants in signal transduction and gene expression. Nutr Rev 1997: 55: 353–61.
10. Frank L, Massaro D. Oxygen toxicity. Am J Med 1980: 69: 117–26.
11. Ames BN, Shigenda MK, Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. Proc Natl Acad Sci USA 1993: 90: 7915–22.
12. Edgington SM. As we live and breathe: free radicals and aging. Biotechnology 1994: 12: 37–40.
13. Tanaka M, Gong J-S, Zhang J, Yoneda M, Yagi K. Mitochondrial genotype associated with longevity [research letter]. Lancet 1998: 351: 185–6.
14. Holley AE, Cheeseman KH. Measuring free radical reactions *in vivo*. Br Med Bull 1993: 49: 494–505.
15. Halliwell B, Chirico S. Lipid peroxidation: its mechanism, measurement, and significance. Am J Clin Nutr 1993: 57(Suppl.): 715S–25S.
16. Crow JP, Ischiropoulos H. Detection and quantification of nitrotyrosine residues in proteins: *in vivo* marker of peroxynitrite. Method Enzymol 1996: 269: 185–94.
17. Buss H, Chan TP, Sluis KB, Domigan NM, Winterbourn CC. Protein carbonyl measurement by a sensitive ELISA method. Free Rad Biol Med 1997: 23: 361–6.
18. Morrow JD, Hill KE, Burk RF, Nammour TM, Badr KF, Roberts II LJ. A series of prostaglandin F2-like compounds are produced *in vivo* in humans by a non-cyclooxygenase, free radical-catalysed mechanism. Proc Natl Acad Sci USA 1990: 87: 9383–7.
19. Delanty N, Reilly M, Pratico D, Fitzgerald DJ, Lawson JA, Fitzgerald GA. 8-epi-$PGF_{2\alpha}$: Specific analysis of an isocicosanoid as an index of oxidant stress *in vivo*. Brit J Clin Pharmacol 1996: 42: 15–9.
20. Helbock HJ, Beckman KB, Shigenaga MK et al. DNA oxidation matters: the HPLC-electrochemical detection assay of 8-oxo-deoxyguanosine and 8-oxo-guanine. Proc Natl Acad Sci 1998: 95: 288–93.
21. Halliwell B. Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? Lancet 1994: 344: 721–4.
22. Hennekens CH, Buring JE, Peto R. Antioxidant vitamins – benefits not yet proved. N Engl J Med 1994: 330: 1080–1.
23. Greenberg ER, Sporn MB. Antioxidant vitamins, cancer, and cardiovascular disease. N Engl J Med 1996: 334: 1189–90.
24. Steinberg D, Parathasarathy S, Carew TE, Khoo JC, Witztum JL. Beyond cholesterol: modification of low density lipoprotein that increase its atherogenicity. N Engl J Med 1989: 320: 915–24.
25. Salonen JT, Ylä-Herttuala S, Yamamoto R et al. Autoantibody against oxidised LDL and progression of carotid atherosclerosis. Lancet 1992: 883–7.
26. Pratico D, Iuliano L, Mauriello A et al. Localization of distinct F_2-isoprostanes in human atherosclerotic lesions. J Clin Invest 1997: 100: 2028–34.
27. Salonen JT, Nyyssonen K, Salonen R et al. Lipoprotein oxidation and progression of carotid atherosclerosis. Circulation 1997: 95: 840–45.
28. Kritchevsky SB, Shimakawa T, Tell GS et al. Dietary antioxidants and carotid artery wall thickness. The ARIC study. Circulation 1995: 92: 2142–50.
29. Bonithon-Kopp C, Coudray C, Berr C et al. Combined effects of lipid peroxidation and antioxidant status on carotid atherosclerosis in a population aged 59–71 y: the EVA Study. Am J Clin Nutr 1997: 65: 121–7.
30. Major Ongoing Stroke Trials. Stroke 1998: 29: 552.
31. Keli SO, Hertog MGL, Feskens EJM, Kromhout D. Dietary flavonoids, antioxidant vitamins, and incidence of stroke. Arch Intern Med 1996: 154: 637–42.
32. Pulsinelli W. Pathophysiology of acute ischaemic stroke. Lancet 1992: 339: 533–6.
33. Delanty N, MacGowan D, Reilly M, Lawson J, FitzGerald G. Measurement of the free radical derived isoprostane, 8-epi–prostaglandin $F_{2\alpha}$, in acute stroke (abstract). J Stroke Cerebrovasc Dis 1997: 6: 152–3.
34. The RANTTAS Investigators. A randomized trial of tirilazad mesylate in patients with acute stroke (RANTTAS). Stroke 1996: 27: 1453–8.
35. Reilly MP, Delanty N, Lawson JA, FitzGerald GA. Modulation of oxidant stress *in vivo* in chronic cigarette smokers. Circulation 1996: 94: 19–25.
36. Sharma RC, Hodis HN, Mack WJ, Sevanian A, Kramsch DM. Probucol suppresses oxidant stress in hypertensive arteries. Immunohistochemical evidence. Am J Hypertens 1996: 9: 577–90.
37. Davi G, Alessandrini P, Mezzetti A et al. *In vivo* formation of 8-epi-prostaglandin $F_{2\alpha}$ is increased in hypercholesterolemia. Arterioscler Thromb Vasc Biol 1997: 17: 3230–5.
38. McCord JM. Oxygen-derived free radicals in postischemic tissue injury. N Engl J Med 1985: 312: 159–63.
39. Delanty N, Reilly MP, Pratico D et al. 8-epi Prostaglandin $F_{2\alpha}$ generation during coronary reperfusion. A potential quantitative marker of oxidant stress *in vivo*. Circulation 1997: 95: 2492–9.
40. Reilly MP, Delanty N, Roy L et al. Increased formation of the isoprostanes $IPF_{2\alpha}$-I and 8-epi-prostaglandin $F_{2\alpha}$ in acute coronary angioplasty. Evidence for oxidant stress during coronary reperfusion in humans. Circulation 1997: 96: 3314–20.
41. Hall ED, McCall JM, Means ED. Therapeutic potential of the lazaroids (21-aminosteroids) in acute central nervous system trauma, ischemia and subarachnoid hemorrhage. Adv Pharmacol 1994: 28: 221–68.
42. Hoffman SW, Moore S, Ellis EF. Isoprostanes: free radical-generated prostaglandins with constrictor effects on cerebral arterioles. Stroke 1997: 28: 844–9.

43. Polidori MC, Frei B, Kordorf G, Ogilvy CS, Koroshetz WJ, Beal MF. Increased levels of plasma cholesterol ester hydroperoxides in patients with subarachnoid hemorrhage. Free Rad Biol Med 1997: 23: 762–7.
44. Kassell NF, Haley Jr EC, Apperson-Hansen C, Alves WM, and the participants. Randomized, double-blind, vehicle-controlled trial of tirilazad mesylate in patients with aneurysmal subarachnoid hemorrhage: a cooperative study in Europe, Australia, and New Zealand. J Neurosurg 1996: 84: 221–8.
45. Haley Jr EC, Kassell NF, Apperson-Hansen C, Maile MH, Alves WM, and the participants. A randomized, double-blind, vehicle-controlled trial of tirilazad mesylate in patients with aneurysmal subarachnoid hemorrhage: a cooperative study in North America. J Neurosurg 1997: 86: 467–74.
46. Markesbery WR. Oxidative stress hypothesis in Alzheimer's disease. Free Rad Biol Med 1997: 23: 134–47.
47. Smith MA, Sayre LM, Monnier VM, Perry G. Radical AGEing in Alzheimer's disease. Trends Neurosci 1995: 18: 172–6.
48. Sano M, Ernesto C, Thomas RG et al, and the Alzheimer's Disease Cooperative Study. A controlled trial of selegiline, alpha-tocopherol, or both as treatment for Alzheimer's disease. N Engl J Med 1997: 336: 1216–22.
49. Oyama Y, Chikahisa L, Ueha T, Kanemaru K, Noda K. Ginkgo biloba extract protects brain neurons against oxidative stress induced by hydrogen peroxide. Brain Res 1996: 712: 349–52.
50. Le Bars PL, Katz MM, Berman N, Itil TM, Freedman AM, Schatzberg AF; for the North American EGb Study Group. A placebo-controlled, double-blind, randomized trial of an extract of Ginkgo Biloba for dementia. JAMA 1997: 278: 1327–32.
51. Dana Consortium on the Therapy of HIV Dementia and Related Cognitive Disorders. Safety and tolerability of the antioxidant OPC-14117 in HIV-associated cognitive impairment. Neurology 1997: 49: 142–6.
52. Rosen DR, Siddeque T, Patterson D et al. Mutations in the Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature 1993: 362: 59–62.
53. Gurney M. The use of transgenic mouse models of amyotrophic lateral sclerosis in preclinical drug studies. J Neurol Sci 1997: 152 (Suppl. 1): S67–S73.
54. Gurney ME, Pu H, Chiu AY et al. Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. Science 1994: 264: 1772–5.
55. Brown Jr RH. Superoxide dismutase and familial lateral sclerosis: new insights into mechanisms and treatments. Ann Neurol 1996: 39: 145–6.
56. Gurney ME, Cutting FB, Zhai P et al. Benefit of vitamin E, riluzole, and gabapentin in a transgenic model of familial amyotrophic lateral sclerosis. Ann Neurol 1996: 39: 147–57.
57. Shaw PJ, Ince PG, Falkous G, Mantle D. Oxidative damage to protein in sporadic motor neuron disease spinal cord. Ann Neurol 1995: 38: 691–5.
58. Przedborski S, Donaldson D, Jakowec M et al. Brain superoxide dismutase, catalase, and glutathione peroxidase activities in amyotrophic lateral sclerosis. Ann Neurol 1996: 39: 158–65.
59. Beal MF, Ferrante RJ, Browne SE, Matthews RT, Kowall NW, Brown Jr RH. Increased 3-nitrotyrosine in both sporadic and familial amyotrophic lateral sclerosis. Ann Neurol 1997: 42: 646–54.
60. Delanty N, Przedborski S, Bandele AN, Lynch T, Triplett RR. Elevated protein nitrotyrosine immunoreactivity in patients with amyotrophic lateral sclerosis [abstract]. Ann Neurol 1997: 42: 442–3.
61. Louwerse ES, Wevverling GJ, Bossuyt PMM, Meyjes FEP, Vianney de Jong JMB. Randomized, double-blind, controlled trial of acetylcysteine in amyotrophic lateral sclerosis. Arch Neurol 1995: 52: 559–64.
62. Comi GP, Bordoni A, Salani S et al. Cytochrome c oxidase subunit 1 microdeletion in a patient with motor neuron disease. Ann Neurol 1998: 43: 110–16.
63. Schwartkroin PA. Origins of the epileptic state. Epilepsia 1997: 38: 853–8.
64. Willmore LJ, Hiramatsu M, Kochi H, Mori A. Formation of superoxide radicals after FeCl3 injection into rat isocortex. Brain Res 1983: 277: 393–6.
65. Yokoi I, Toma J, Liu J, Kabuto H, Mori A. Adenosines scavenged hydroxyl radicals and prevented posttraumatic epilepsy. Free Radic Biol Med 1995: 19: 473–9.
66. Singh R, Pathak DN. Lipid peroxidation and glutathione peroxidase, glutathione reductase, superoxide dismutase, catalase, and glucose-6-phosphate dehydrogenase activities in FeCl3-induced epileptogenic foci in the rat brain. Epilepsia 1990: 31: 15–26.
67. Willmore LJ, Rubin JJ. Antiperoxidant pretreatment and iron-induced epileptiform discharges in the rat: EEG and histopathologic studies. Neurology 1981: 31: 63–9.
68. Ratan RR, Murphy TH, Barban JM. Oxidative stress induces apoptosis in embryonic cortical neurons. J Neurochem 1994: 62: 376–9.
69. Ferrari G, Yan CYI, Greene LA. *N*-Acetylcysteine (D- and L- stereoisomers) prevents apoptotic death of neuronal cells. J Neurosci 1995: 15: 2857–66.
70. Bagetta G, Palma E, Piccirilli S, Nistico G, Dolly, JO. Seizures and hippocampal damage produced by dendrotoxin-K in rats is prevented by the 21-aminosteroid U-74389G. Exp Neurol 1997: 147: 204–10.
71. Ogunmekan AO, Hwang PA. A randomized, double-blind, placebo-controlled, clinical trial of D-α-tocopherol acetate (vitamin E), as add-on therapy, for epilepsy in children. Epilepsia 1989: 30: 84–9.
72. Hurd RW, Wilder BJ, Helveston WR, Uthman BM. Treatment of four siblings with progressive myoclonus epilepsy of the Unverricht-Lundborg type with *N*-acetylcysteine. Neurology 1996: 47: 1264–8.
73. Ramaekers VT, Calomme M, Vanden Berghe D, Makropoulos W. Selenium deficiency triggering intractable seizures. Neuropediatrics 1994: 25: 217–23.
74. Maertens P, Dyken P, Graf W, Pippenger C, Chronister R, Shah A. Free radicals, anticonvulsants, and the neuronal ceroid-lipofuscinoses. Am J Med Genet 1995: 57: 225–8.
75. Sofic E, Paulus W, Jellinger K, Riederer P, Youdim MBH. Selective increase of iron in substantia nigra zona compacta of parkinsonian brains. J Neurochem 1991: 56: 978–82.
76. Sian J, Dexter DT, Lees AJ et al. Alterations in glutathione levels in Parkinson's disease and other neurodegenerative disorders affecting basal ganglia. Ann Neurol 1994: 36: 348–55.
77. Wei Q, Yeung M, Jurma OP, Anderson JK. Genetic elevations of monoamine oxidase levels in dopaminergic PC12 cells results in increased free radical damage and sensitivity to MPTP. J Neurosci Res 1996: 46: 666–73.
78. Cohen G. The brain on fire. Ann Neurol 1994: 36: 333–4.
79. Nakao N, Frodl EM, Duan W-M, Widner H, Brundin P. Lazaroids improve the survival of grafted rat embryonic dopamine neurons. Proc Natl Acad Sci 1994: 91: 12408–12.
80. Nakao N, Frodl EM, Widner H et al. Overexpressing Cu/Zn superoxide dismutase enhances survival of transplanted neurons in a rat model of Parkinson's disease. Nat Med 1995: 1: 226–31.
81. The Parkinson's Disease Study Group. Effects of toco-

pherol and deprenyl on the progression of disability in early Parkinson's disease. N Engl J Med 1993: 328: 176–83.
82. May JM, Qu Z-C, Mendiratta S. Protection and recycling of α-tocopherol in human erythrocytes by intracellular ascorbic acid. Arch Biochem Biophys 1998: 349: 281–9.
83. Browne SE, Bowling AC, MacGarvey U et al. Oxidative damage and metabolic dysfunction in Huntington's disease: selective vulnerability of the basal ganglia. Ann Neurol 1997: 41: 646–53.
84. Peyser CE, Folstein M, Chase GA et al. Trial of D-α-tocopherol in Huntington's disease. Am J Psychiatry 1995: 152: 1771–5.
85. Ranen NG, Peyser CE, Coyle JT et al. A controlled trial of idebenone in Huntington's disease. Mov Disord 1996: 11: 549–54.
86. Gotoda T, Arita M, Arai H et al. Adult-onset spinocerebellar dysfunction caused by a mutation in the gene for the α-tocopherol-transfer protein. N Engl J Med 1995: 333: 1313–18.
87. Yokota T, Shiojiri T, Gotoda T, Arai H. Retinitis pigmentosa and ataxia caused by a mutation in gene for the α-tocopherol transfer protein [letter]. N Engl J Med 1996: 335: 1770–1.
88. Shohami E, Beit-Yanni E, Horowitz M, Kohen R. Oxidative stress in closed head injury: brain antioxidant capacity as an indicator of functional outcome. J Cereb Blood Flow Metab 1997: 1007–19.
89. Bracken MB, Shepard MJ, Holford TR, et al; for the National Acute Spinal Cord Injury Study. Administration of methylprednisolone for 24 or 48 hours or tirilazad mesylate for 48 hours in the treatment of acute spinal cord injury. JAMA 1997: 277: 1597–604.
90. Marshall LF, Marshall SB, Musch B, Means E. Outcome of moderate to severe head injury in patients treated with tirilazad mesylate (abstract). J Neurosurg 1996: 84: 342A.